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Correlation between Neurotoxic Esterase Inhibition and Mipafox-Induced Neuropathic Damage in Rats

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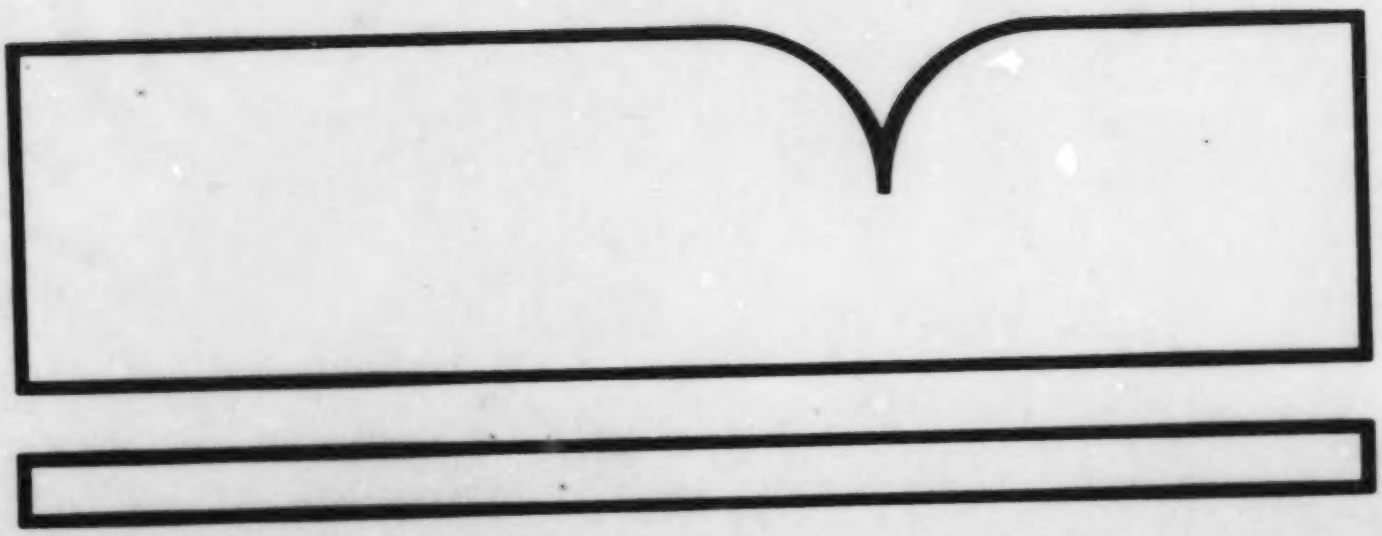

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JOURNAL ARTICLE

The Correlation Between Neurotoxic Esterase Inhibition and Mipafox-Induced Neuropathic Damage in Rats

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ABSTRACT: *The correlation between neuropathic damage and inhibition of neurotoxic esterase or neuropathy target enzyme (NTE) was examined in rats acutely exposed to Mipafox (N, N'-diisopropylphosphorodiamidofluoridate), a neurotoxic organophospate. Brain and spinal cord NTE activities were measured in Long-Evans male rats 1 hr post-exposure to various dosages of Mipafox (ip, 1-15 mg/kg). These data were correlated with histologically scored cervical cord damage in a separate group of similarly dosed rats sampled 14-21 days post-exposure. Those dosages (≥ 10 mg/kg) that inhibited mean NTE activity in the spinal cord ≥ 73% and brain ≥ 67% of control values produced severe (≥ 3) cervical cord pathology in 85% of the rats. In contrast, dosages of Mipafox (≤ 5 mg/kg) which inhibited mean NTE activity in spinal cord ≤ 61% and brain ≤ 60% produced this degree of cord damage in only 9% of the animals. These data indicate that a critical percentage of NTE inhibition in brain and spinal cord sampled shortly after Mipafox exposure can predict neuropathic damage in rats several weeks later.*

Key Words: Neurotoxic Esterase, Mipafox, OPIDN, Rodent Model of OPIDN

INTRODUCTION

Depression of the putative target enzyme activity, neurotoxic esterase or neuropathy target enzyme (NTE) (Johnson, 1969), by >65% shortly after exposure to neurotoxic organophosphorus compounds (OP) heralds the onset of neuropathic damage 14-21 days later in experimental animals (Johnson, 1975b). The relationship between this critical percentage of NTE inhibition and the delayed neuropathy (OPIDN) associated with neurotoxic OP's has been validated in the chicken for hundreds of *in vitro* and *in vivo* tested compounds (Johnson, 1969, 1970, 1975a,b; Lotti and Johnson, 1978; Ohkawa *et al.*, 1980) and is considered a reliable indicator of the delayed neuropathy (Johnson, 1982). Recent studies have demonstrated that the rat, previously thought to be neurologically insensitive to OPIDN, is only functionally refractory, and develops, after acute OP exposure, a neuropathic profile (*i.e.*, distal "dying back" axonopathy) similar to that seen in more conventional animal models of OPIDN (*e.g.*, chickens, cats) (Veronesi, 1984). Recently, we reported that a mean NTE inhibition of > 65% in rat brain and spinal cord could predict severe spinal cord neuropathy in rats orally exposed to tri-ortho-cresyl phosphate (TOCP) (Padilla and Veronesi,

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1985). Our present experiment was designed to determine if a similar threshold of inhibition would predict pathology in rats treated with a neurotoxic organophosphate which, unlike TOCP (Casida *et al.*, 1963), does not require metabolic activation for neurotoxic potency. The results of this study, coupled with evidence of interspecies neuropathic similarities, strengthens the likelihood that the rat may substitute for the chicken in the testing of delayed organophosphate neurotoxicants.

MATERIALS AND METHODS

Chemicals. Bovire serum albumin (fraction V), atropine sulfate (Sigma Chemical Co., St. Louis, MO) and paraoxon (diethyl p-nitrophenyl phosphate, Aldrich Chemical Co., Milwaukee, WI; ≥ 92% pure) were obtained commercially. Mipafox (*N, N'*-diisopropyl-phosphorodiamidofluoridate; ≥ 98% pure) and phenyl valerate (≥ 99% pure) were synthesized by Dr. Salah Soliman (Alexandria, Egypt).

Animal Care and Dosing. Long-Evans male 60 d rats (Charles River, Inc., Portage, MI) were housed in pairs on sterile bedding and allowed free access to feed (Purina Rat Chow) and water. Animal rooms were kept on a 12 hr alternating light/dark cycle and held at 22°C and 50% relative humidity. Non-fasted animals were dosed contemporaneously for biochemistry and neuropathology with Mipafox (ip, 1, 3, 5, 10, 15 mg/kg) dissolved in saline. All rats including the saline-vehicle controls received prophylactic atropine sulfate (ip, 30 mg/kg) treatment 20 min before and 4 hrs after dosing.

Biochemistry (NTE Assay). NTE activity was determined in rats decapitated 1 hr after Mipafox injection. The brain and cervical spinal cord were removed, weighed and placed in 5 volumes (w/v) of ice-cold buffer (50 mM Tris/HCl, 0.2 mM EDTA, pH 8.0) and a 9K supernatant was prepared (Soliman and Farmer, 1984). The 9K supernatant fraction, rather than the more turbid tissue homogenate, was used for subsequent NTE activity and protein determinations. NTE activity was assayed according to the Johnson technique (1977) with slight modifications (Soliman *et al.*, 1982b) and defined as the difference in phenyl valerate hydrolyzing activity between paired samples of each tissue in the presence of either paraoxon (40 μm) or paraoxon (40 μm) plus Mipafox (50 μm). Protein concentrations were determined according to Lowry *et al.* (1951) using bovine serum albumin as the standard.

NTE activity levels (nmol/min/mg Lowry protein) were used to determine the lowest significant dosage (Williams, 1972) and in the linear regression analysis to correlate brain NTE activity with spinal cord NTE activity across all dosages. Percentage inhibition was calculated by dividing the individual NTE activity for each treated animal by the mean activity value for their respective control group. The linear regression lines produced by comparison of Mipafox dosage versus percentage NTE inhibition in brain and spinal cord were tested for equality to one another according to the multivariate regression test of equality of lines (SAS Institute, Inc., 1982).

Neuropathology (Tissue Preparation). Rats from each treatment group (n = 5-10) were killed 14-21 days post-exposure. Animals were anesthetized (ip) with 50 mg/kg sodium pentobarbital (Abbott Laboratories, Chicago, IL) containing 1% heparin (50 units/ml) prior to vascular perfusion with Sorenson's buffered 5% glutaraldehyde. The upper cervical spinal cord (C2) was removed, post-fixed in Dalton's 2% osmium tetroxide, dehydrated in increasing grades of ethanol, cleared in propylene oxide and embedded in epoxy resin. Three semi-thin (1.0 micrometer) epoxy sections were cut from the cervical cord and stained with 1% Toluidine blue-o in 1% sodium borate. Areas of interest were photographed with a Leitz Ortholux microscope using Kodak Technical Pan Film 2415 (ASA 50). Thin (60-70nm) sections (based on interference colors) were cut, stained with uranyl acetate and lead citrate, and photographed with a Phillips 410 electron microscope.

Analysis of Cervical Cord Damage. The fasciculus gracilis of the cervical cord was scored 0-4 by previously defined criteria (Padilla and Veronesi, 1985). Briefly, a damage score of 0 = no fiber degeneration or myelin debris noted; 1 = individual or several (2-3) swollen/degenerated axons noted; 2 =

scattered groups or swollen axons or narrow rim of degeneration seen along the fasciculus gracilis; 3 = more lateral and wider spread of fascicular degeneration; and 4 = funnel-shaped pattern of severe degeneration involving most of the fasciculus gracilis. All three cervical sections were read blind and individually scored. The highest score on each animal was pooled according to dosage group.

RESULTS

General Condition of the Animals

Within 30-60 min post-exposure, rats treated with 10 or 15 mg/kg Mipafox exhibited severe tremors which continued for 48 hr. The intensity of the tremors was alleviated but never totally eliminated by atropine sulfate. The rats appeared agitated and were easily startled by loud sounds or sudden motions. Cholinergic signs (*e.g.*, lacrimation, salivation, and diarrhea) were noted in ~10% of the higher dosed animals and a 25% mortality rate was recorded for these groups. Rats treated with 1, 3, or 5 mg/kg Mipafox appeared subdued for 36-48 hrs post-exposure, but never developed the tremors or other cholinergic signs noted in the higher dosed animals. No mortality occurred in these groups.

Biochemistry

Control values for NTE activity were 10.1 ± 0.6 and 8.4 ± 0.6 nmol phenyl valerate hydrolyzed/min/mg Lowry protein in brain and cervical spinal cord, respectively. Table 1 delineates NTE inhibition by test group. In general, NTE inhibition for both brain and spinal cord followed a tight dose-response pattern (Fig. 1). Linear regression lines fitted to the dose-response data for NTE inhibition resulted in a highly significant ($p \leq 0.001$) dose related inhibition in both brain and spinal cord. The slopes and intercepts of the regression lines for the brain and spinal cord dose response data did not differ significantly ($p \geq 0.67$) from one another. According to the Williams test for significant doses (Williams, 1972), all the dosages of Mipafox produced significant depression in brain and spinal cord NTE activity compared to control values ($p \leq 0.05$). A least square linear regression analysis indicated that at all dosage levels, brain NTE activity levels reflected spinal cord NTE activity levels. The linear regression line had a slope of 0.8, an intercept of 0.0 and $r^2 = 0.76$.

Neuropathology

In rats exposed to Mipafox (10 and 15 mg/kg), advanced degeneration, consisting of hyaline bodies, myelin ellipsoids, fiber debris and giant axonal swellings, was seen in the fasciculus gracilis (Fig. 2). Two varieties of axonal pathology were noted in the cervical cord. One type (*i.e.*, the tubulovesicular axonal swelling) was swollen with accumulated tubular profiles penetrated by channels of undisrupted mitochondria (FIGS. 3a, 3b). Another type of axonal damage (*i.e.*, vacuolar axonal pathology), which was rarely seen in the lower dosed rats, occured with the tubulovesicular swelling and consisted of fibers whose axoplasm was compressed to one side of the axolemmal by an intra-axonal vacuole(s) (Fig. 4). Both types of axonal changes were used to score the cervical cord damage (see below).

TABLE 1. Relationship Between NTE Inhibition and Cervical Cord Pathology[a]

Mipafox Dosage (MG/KG)	Mean NTE Inhibition (1 hr) Brain	Cervical Cord	Mean Damage Score	% Showing Damage ≥3
0	0	0 (12)[b]	0.86 ± .38 (8)	0%
1	29.5 ± 5.9%	28.0 ± 4.8% (3)	1.00 ± .29 (10)	10%
3	36.1 ± 6.2%	28.4 ± 13.1% (3)	1.00 ± .25 (10)	0%
5	60.5 ± 2.3%	61.3 ± 6.0% (9)	1.50 ± .42 (6)	16%
10	66.7 ± 6.0%	73.1 ± 6.2% (7)	2.60 ± .24 (5)	60%
15	91.8 ± 3.0%	89.9 ± 1.3% (3)	3.37 ± .18 (8)	100%

[a] Data obtained from different sets of rats for NTE inhibition and cervical pathology.
[b] Numbers in parentheses indicate the number of sampled animals.

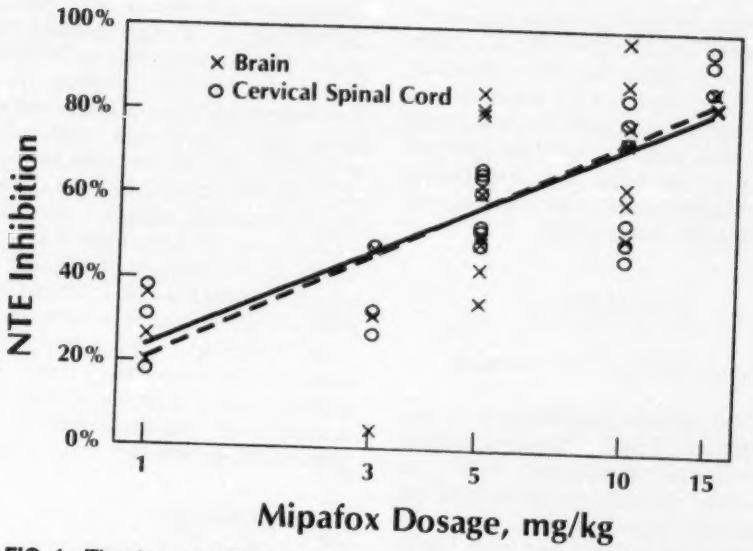

FIG. 1. The degree of NTE inhibition in response to Mipafox treatment (1-15 mg/kg) is shown for individual animal brain (X) and spinal cord (0) samples 1 hr post-dosing. The linear relationship between tissue NTE inhibition and Mipafox dosage can be expressed as follows: Brain NTE inhibition = 0.242 + 0.481 log (Mipafox dose); Spinal cord NTE inhibition = 0.214 + 0.519 log (Mipafox dose). These lines are drawn on the graph for brain (——) and for spinal cord (-----).

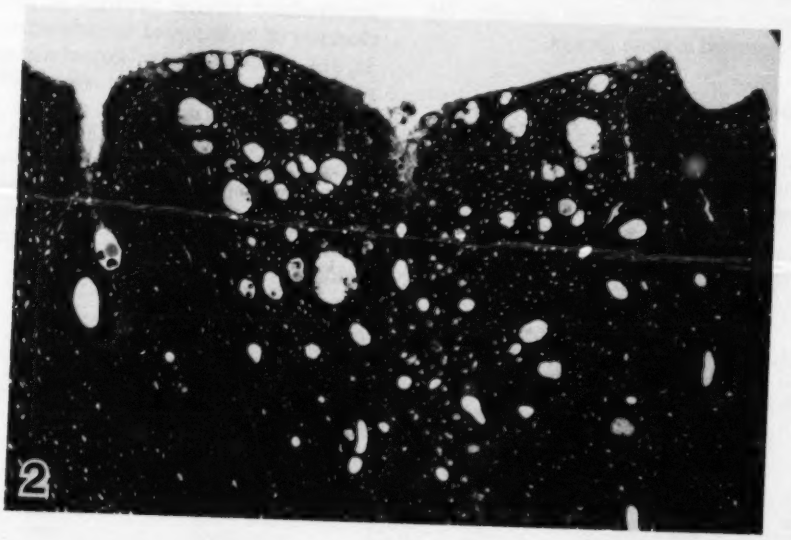

FIG. 2. Epoxy section of cervical cord from Mipafox (10 mg/kg) treated rat showing extensive (3) neuropathic damage consisting of vacuolated swollen axons, myelin ellipsoids, fiber debris and giant axonal swellings. X 664.

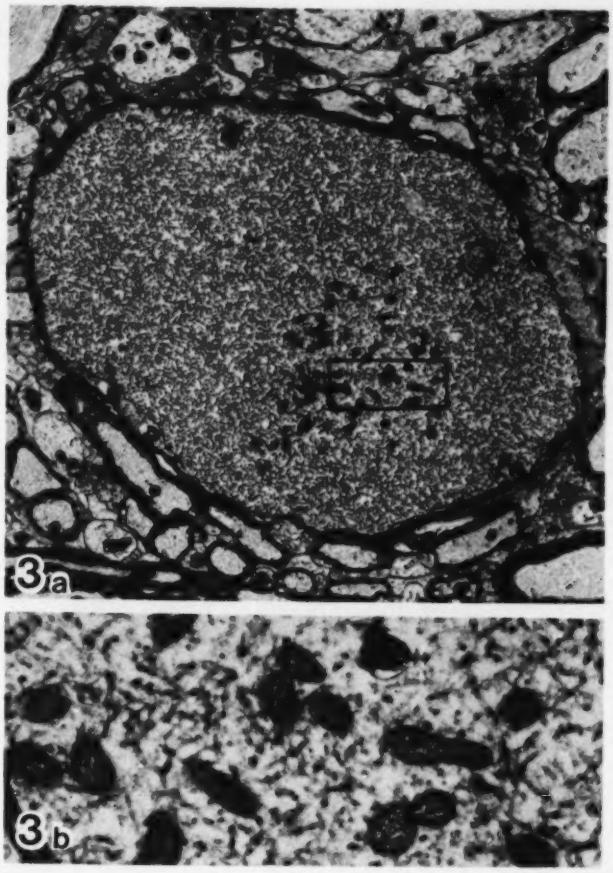

FIG. 3. (A) The tubulovesicular axonal swelling distended with agranular endoplasmic reticulum, tubular profiles and vesicles hallmarked the neuropathology of Mipafox-treated rats. X 7,024. (B) Higher magnification of enclosed area (FIG. 3A) shows masses of interlaced tubulovesicular profiles. X 32,000.

Cervical Cord Damage

The data are presented in both scattergram and bar graph form with the means ± SE (Fig. 5). As with NTE inhibition, mean cervical cord damage scores followed a dose-response pattern. Although the damage scores on rats dosed with 1, 3, or 5 mg/kg did not differ statistically from each other, they did differ from the 10 and 15 mg/kg dosed animals. As in a previous study relating cervical cord damage to NTE inhibition in TOCP-exposed rats (Padilla and Veronesi, 1985), a damage score of "3", indicating a wide, lateral spread of fascicular degeneration, was used as the numerical end-point of definitive cervical damage. Using this criterion, only 9% of all rats dosed with 1, 3 or 5 mg/kg Mipafox ($n = 32$) showed scores of ≥ 3. In contrast, over 85% of the rats dosed with 10 or 15 mg/kg Mipafox ($n = 13$) showed damage scores ≥ 3.

FIG. 4. Expansion of intra-axonal vacuoles in the vacuolar axonal swelling compressed the axoplasm into a membrane-bordered rim. X 44,818.

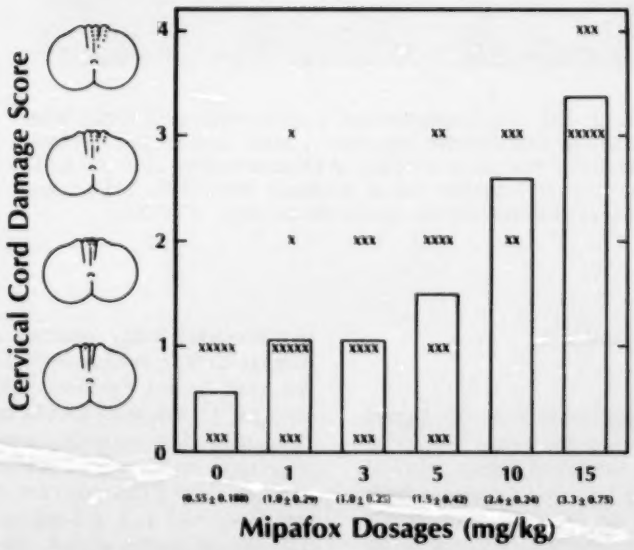

FIG. 5. Scoring of cervical cord neuropathy. Data are presented in scattergram and bar graph form, with means ± SE. Calculations are explained in text.

Correlation of NTE Inhibition and Cord Damage

The relationship between NTE inhibition and definitive (≥ 3) spinal cord damage is shown in Table 1. These data, derived from separate groups of animals, indicate that dosages of Mipafox (10 and 15 mg/kg) which depressed mean brain NTE activity $\geq 67\%$ and spinal cord NTE activity $\geq 73\%$ produced severe (≥ 3) spinal cord pathology in 85% of similarly exposed rats. Dosages of Mipafox (1, 3, 5 mg/kg) which depressed cervical cord NTE activity $\leq 61\%$ and brain NTE activity $\leq 60\%$ produced spinal cord damage (≥ 3) in 9% of the total population of similarly treated animals.

DISCUSSION

The relationship between a critical percentage (> 65%) of NTE inhibition and OPIDN has been demonstrated most frequently in the chicken using both *in vitro* (Johnson, 1975a,b; Ohkawa *et al.*, 1980) and *in vivo* (Johnson, 1970) assays with brain and spinal cord as tissue sources. These data have provided a quantifiable means of predicting neurotoxic hazard for hundreds of tested OP compounds. Similarly, we have demonstrated that brain NTE inhibition of > 65% predicts pathology in rats exposed to those OP's that inhibit NTE directly (*e.g.*, Mipafox) or are dependent upon bioactivation for neurotoxic potency (*e.g.*, TOCP). The time required to inhibit NTE maximally with "equipathic" doses of a direct versus an indirect acting OP varies in the rat. In rats orally exposed to TOCP at doses (1160-3480 mg/kg) which produces scored cervical damage of ≥ 3 in $\geq 90\%$ of the animals, a maximal NTE inhibition of 90% is achieved by 44 hr post-exposure (Padilla and Veronesi, 1985). In the present study, maximal inhibition of 90-92% was achieved 1 hr post-exposure to 15 mg/kg Mipafox, a dose which produced definitive cervical cord damage (≥ 3) in 100% of the rats. The difference in time needed for maximal NTE inhibition may be related to the metabolic dependence of TOCP for neurotoxic bioactivation. Intragroup variability of NTE inhibition is also higher in TOCP versus Mipafox-treated rats. Mathematically this variability can be expressed as the coefficient of variation (*i.e.*, the standard deviation/mean); the higher the coefficient of variation, the higher the variability of the observations. After 1 hr post-exposure to Mipafox, rat brain NTE activity had a coefficient of variation that was one half that reported in TOCP-treated rats. Again, the metabolic dependence of TOCP may contribute to the noted intragroup variability.

The time course of NTE activity in rats exposed to Mipafox and chickens exposed to diisopropyl phosphorofluoridate (DFP), another direct-acting OP, indicates interspecies differences in NTE's recovery profile. Neither Mipafox nor DFP are dependent on bioactivation for neurotoxic potency (Clothier and Johnson, 1979; Soliman *et al.*, 1982a). Both OP's contain a hydrolyzable R-O(NH)-P bond and thus are capable of "aging" (Clothier and Johnson, 1980; Williams, 1983). After acute exposure, both of these chemical analogues produce the hindlimb paralysis associated with OPIDN in chickens (Majno and Karnovsky, 1961; Davies *et al.*, 1966; Olajos *et al.*, 1978; Carrington and Abou-Donia, 1983), and both produce delayed neuropathic damage in the rat (*i.e.*, degeneration of the large ascending and descending spinal cord tracts) (Veronesi, unpublished data). Data from a companion study (Veronesi and Padilla, 1985) report that in rats exposed (ip) to 15 mg/kg Mipafox, brain NTE is inhibited by 90% at 4 hr post-exposure and recovers to 50% activity within 2 days. Data from a similar study (Carrington and Abou-Donia, 1984) report that in chickens exposed (sc) to 1.7 mg/kg DFP, peripheral nerve NTE is inhibited 91% at 4 hr post-exposure but requires 7 days to recover to 50% activity. This 5-7 day half-life for NTE recovery has also been demonstrated in the chicken brain subsequent to DFP exposure (Johnson, 1975b; Caroldi and Lotti, 1982). This apparent species difference in NTE recovery time may relate to different rates of replenishing NTE and could explain the rat's resistance to OP-induced ataxia, since prolonged NTE inhibition may be necessary to precipitate dysfunction.

A perplexing feature of NTE centers on the rostral-caudal gradient of baseline NTE activity (viz. the brain contains higher net NTE activity than spinal cord or peripheral nerves) that is seen in all tested species (Dudek and Richardson, 1982; Johnson, 1982). Human autopsy material and nerve biopsy samples

indicate that a similar gradient occurs in man with the spinal cord containing one-third of the cortical activity and the peripheral nerves containing low but significant activity (Lotti and Johnson, 1980). This observation is a curiosity to the overall picture of OPIDN since the brain, which contains higher NTE activity per gram weight than spinal cord, is relatively free of neuropathic damage compared to the cord and peripheral nerves. Although a gradient of baseline activity has been established across species, NTE is inhibited and recovers uniformly throughout the neuraxis (Caroldi and Lotti, 1981, 1982). A recent paper (Carrington and Abou-Donia, 1984) however, argues that NTE recovery in the distal segment of chicken sciatic nerve is delayed relative to recovery in the proximal length, an observation which may explain the susceptibility of the distal site to neuropathic damage.

In summary, our results indicate that dose-related NTE inhibition of > 65% sampled 1 hr post-exposure predicts neuropathic damage several weeks later in Mipafox-treated rats. Coupled with previous findings relating NTE inhibition and TOCP-induced neuropathy, it appears that the degree of NTE depression necessary to precipitate neurological damage in chickens, cats and farm animals is shared by the rat and underscores its biochemical similarity to other experimental models of OPIDN.

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This manuscript has been reviewed by the Health Effects Research Laboratory, US EPA, and is approved for publication. Mention of trade names and commercial products does not constitute endorsement or recommendation for their use.

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